# HORMONAL MANIPULATION OF DIGESTIVE ENZYME ONTOGENY IN MARINE LARVAL FISHES - EFFECTS ON DIGESTIVE ENZYMES

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## ABSTRACT

It has been demonstrated previously that the ontogeny of digestive enzymes in the Pacific threadfin *Polydactylus sexfilis* (locally known as *moi*) follows a pattern in which amylase is the first to become activated, followed by lipase and protease later in development. The timing of digestive enzyme development suggests a greater importance of carbohydrates during the early, critical, period of first feeding than might be expected for carnivorous larval fishes.

Treatment of larval threadfin with a combination of hormones including triiodothyronine and cortisol has been shown to improve survival in this species, at least in part by advancing the timing of initial intestinal absorptive function. A single, brief (1-h) exposure to the same hormones at hatching also advances the pattern of expression of intestinal enzyme patterns at the onset of feeding. Although the advance is only by a matter of a few hrs, it does appear to cause the increase in specific activities of amylase and serine protease throughout the experimental period. This suggests that these enzymes are inducible, characteristically appearing around the time of first feeding. The hormone-dependent increase in digestive capacity coincident with first feeding may improve nutrient utilization, and may therefore be one mechanism by which exposure to these development-promoting hormones increases larval survival.

Starved larvae undergo a decrease in the production of amylase at approximately the latest time that feeding can begin (termed the *point of no return*; Blaxter 1969). These results suggest that amylases and their products may be of importance in early larval differentiation, and indicate a key role of hormones in the regulation of their expression.

## INTRODUCTION

Many regulatory compounds such as hormones, neurohormones, neurotransmitters, and mRNAs which encode for growth factors and other compounds are maternally derived and deposited into the yolk of vertebrate eggs (reviewed by Brown and Núñez 1994). These regulatory compounds are particularly important in ontogenetic development and initiation of function in larval organ systems. It is possible that the rates of deposition of these regulatory compounds can vary, and that they may be affected by artificial diets and other environmental parameters to which broodstock female fish are subjected. Though hatchery techniques have improved steadily over recent years, early mortality at first feeding remains a primary bottleneck in mass production of marine juvenile fishes.

Thyroid hormones are important regulatory hormones that increase epidermal mitotic rate by controlling the synthesis of specialized proteins during cell differentiation within the digestive system, in the formation and inflation of the swim bladder, and in the development of muscle tissue (Hourdry 1993). They are also reported to frequently have the net effect of improving larval health and survival. Thyroid hormone treatments increased larval survival in tilapia, rabbitfish, striped bass, walleye, goldstriped amberjack, and carp (Lam 1980; Lam and Sharma 1985; Brown et al. 1988, 1989; Miwa et al. 1992; Ayson and Lam 1993; Hey and Farrar 1996; Tachihara et al. 1997). The results from the experimental thyroid hormone treatment of fish

eggs (embryos) and larvae are, however, not conclusive mainly due to the sensitivity to species-specific timing and dosage applied. It is not uncommon that thyroid hormone treatments resulted in altered body morphometrics and overstimulated growth of specific tissues including bones, muscles, and scales (reviewed by Eales 1979).

Thyroid hormones are deposited into eggs against the concentration gradient during vitellogenesis (Norberg et al. 1989; Babin 1992; Bjornsson et al. 1998). Maternally-introduced radioiodine was found in embryonic larvae of coho salmon *Oncorhynchus kisutch* as protein-bound forms (Kobuke et al. 1987), and maternal triiodothyronine ($T_3$) injection elevated $T_3$ level of striped bass *Morone saxitilis* (Brown et al. 1989). Secretion of thyroid hormones varies seasonally (Brown and Stetson 1985), and the uptake of $T_3$ is favored during oogenesis of marine fish eggs. Tagawa (1996), however, demonstrated the possibility of passive diffusion into eggs by estimating the $T_4$ level with which vitellogenin can bind in chum salmon. $T_3$ levels detected in unfertilized eggs of Japanese flounder, barfin flounder, striped jack, and yellowtail were variable between batches, and those levels were not correlated with survival of starved larvae (M. Tagawa, unpublished). Despite unresolved issues in the depository mechanism of thyroid hormones into eggs, those hormones decrease to nearly undetectable levels during the yolk absorption period, suggesting the utilization of thyroid hormones during embryonic development (Tanaka et al. 1995; Tagawa 1996). It has long been established that the period of yolk absorption is a time of sensitivity to thyroid hormones (Brown and Bern 1989). These results imply a role of maternally deposited thyroid hormones in rapidly developing marine embryos and larvae, which may be one determinant of "egg quality." Variation of hormonal levels could contribute to variation in egg quality, which in turn might compromise the consistency of hatchery production. Since hatchery success is largely affected by the survival of larvae through the critical, first-feeding period, egg quality as a consequence of variable maternal deposit may influence such survival.

Another important regulatory hormone in teleosts is cortisol, which is involved in the maintenance of hydromineral balance, osmoregulation, and glucose metabolism (Idler and Truscott 1972; McCormick 1995). Cortisol also acts as a regulator of development throughout the vertebrates. Maternal deposition of cortisol into eggs was evident in Japanese flounder, chum salmon, and tilapia, but cortisol declined to undetectable levels prior to hatching (de Jesus et al. 1991; de Jesus and Hirano 1992; Hwang et al. 1992). Cortisol influenced the timing of hatching in steelhead trout (Yeoh 1993; Mathiyalagan 1996). Cortisol also plays an important role in the regulation of carbohydrate utilization. Cortisol-injected rats had increased amylase activity (Kumegawa et al. 1980) and similar results have been reported in goats and pigs (Sanglid et al. 1994; Lopez et al. 1997). Since amylase activity is elevated during the first half of larval development (Kim 1999; Kim et al. submitted), cortisol may be a particularly important regulatory compound in larval threadfin.

Corticoid and thyroid hormones are known to interact in the regulation of a range of processes of within the target tissues. Cortisol decreased plasma level of $T_3$ in the European eel *Anguilla anguilla* (Redding et al. 1986) and increased hepatic conversion of $T_4$ to $T_3$ in brook char *Salvelinus fontinalis* (Vijayan et al. 1988). During metamorphosis of the Japanese flounder *Paralychthys olivaceus*, resorption of the dorsal-fin rays occurred under the influence of direct peripheral interactions between $T_3$ and cortisol (de Jesus et al. 1990) as found in amphibian metamorphosis (Norris and Dent 1989; Hourdry 1993; Denver 1997). In human premature infants, glucocorticoid and thyroid hormones are commonly used in the treatment of respiratory distress syndrome (Warburton et al. 1988).

In the current study, the effects of treatment with exogenous $T_3$ and cortisol were tested during larval threadfin development under hatchery conditions. Development of the digestive system and particularly the timing of patterns in the digestive enzymes was studied after exposing newly hatched larvae to these hormones by 1-h immersion. A combination of both hormones was administered under identical conditions to the

applications used in previous studies (Brown and Kim 1995; Kim and Brown 1997). A control group was subjected to similar handling, but without hormone exposure.

## MATERIALS AND METHODS

The Pacific threadfin larvae were reared as described in an earlier series of experiments (Brown and Kim 1995; Kim and Brown 1997). All batches of eggs were from the same broodfish which were fed a mixture diet of frozen squid, krill, and artificial pellets while kept in an earthen pond at The Oceanic Institute. Embryonic development indicated that eggs were the products of multiple females, and spawns occurred within 2-4 h.

Newly-hatched larvae were immersed for 1-h in sea water containing a combination of hormone(s); 2.6 ppm of $T_3$ and 0.1 ppm of cortisol (TF group), or untreated sea water serving as a control (C). All larvae for each group were immersed in a bucket containing 12 L of water for each treatment. Following hormone treatment, larvae were stocked into separate rearing tanks (1500-L, fiberglass). The initial stocking density was determined as 40 larvae/L. Additional larvae were kept in a separate tank without live feed supply (starvation) to compare the digestive enzyme activities. The experiment was terminated at the onset of cannibalism (metamorphosis) on d 29 (Kim 1999).

Serial samples of larvae were taken from d 1 after hatching until d 14. More than 1,000 larvae were sampled and specific activities of digestive enzyme activities of whole body tissue were tested as described previously (Kim et al. submitted). In summary, chilled, pooled larval tissues were homogenized in a solution of glycerol saline (Maugle et al. 1982). Following centrifugation, supernatents containing soluble enzymes were divided into aliquots and stored at -70 C until the standard assays for digestive enzymes were performed. The technical aspects of the assays for serine protease, aspartic protease, collagenase, lipase, amylase, chitinase, cellulase, and phosphatase are described in detail by Kim et al. (submitted).

Larvae with induced levels of specific activity (units/mg protein) of digestive enzymes by the combination of $T_3$ and cortisol treatment (TF) were compared with those of untreated larvae (C). The changes in enzyme activities related to hormonal treatment at the time of first feeding were calculated as percentage change occurring between d 0 and d 3 relative to the range of values detected throughout the larval phase for each particular enzyme.

## RESULTS

Among the hormone-treated larvae (TF), specific activities of amylase (Fig.1a), aspartic protease, collagenase, and phosphatase (Fig.1b) were elevated relative to those of untreated larvae by the time of first feeding. After the initiation of feeding, the activities of most digestive enzymes were similar in TF-treated and untreated larvae (Fig.1; d 3.3 or 8 h into d 3). The TF treatment

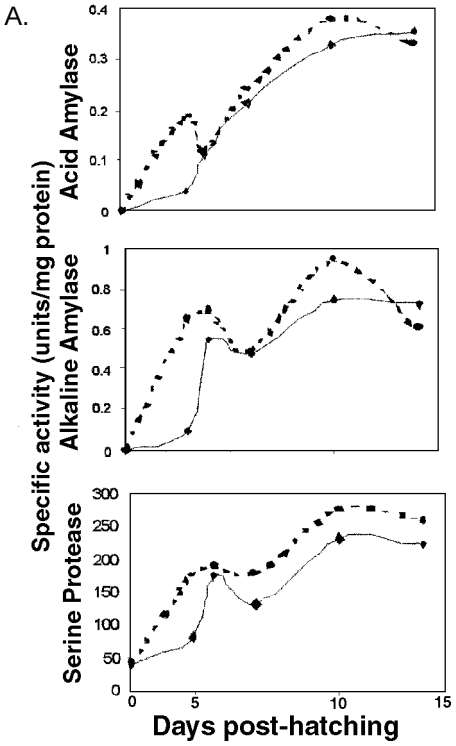
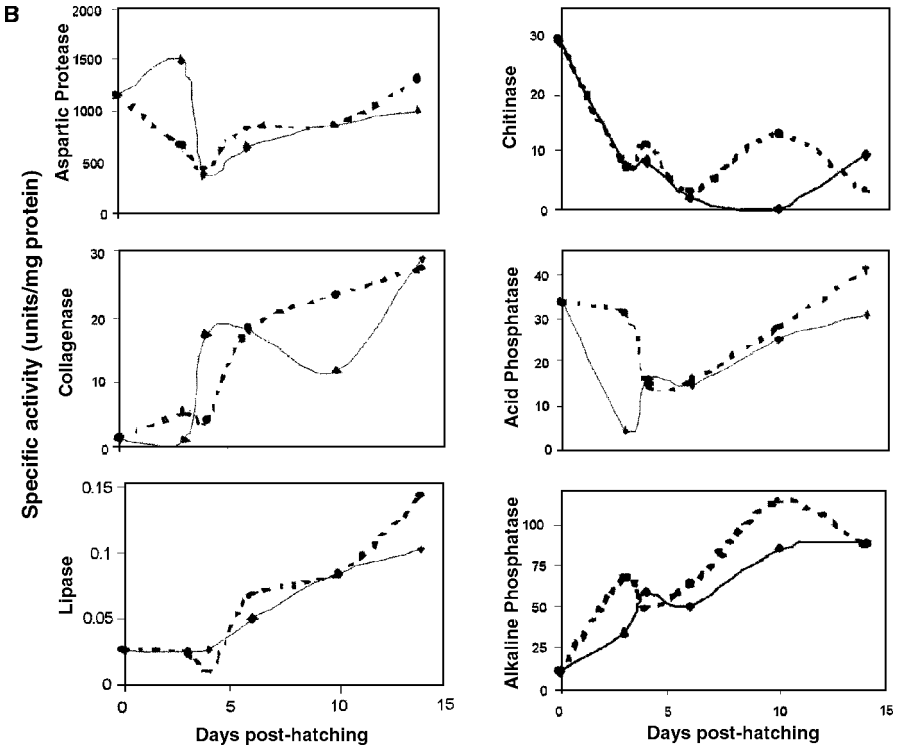



**Figure 1.** Specific activities of digestive enzymes of larval threadfin after treatment with a combination of $T_3$ and cortisol (- - - -) as compared with untreated larvae (——). A. Enzymes that responded to treatment with increased specific activity and B (following page) enzymes that were not affected.




elevated amylase and serine protease activities consistently during the first 2 wk of age (Fig.1 A), while patterns suggesting possible increases in the specific activities of other enzymes were not as clear (Fig.1 B).

The changes in digestive enzyme levels during early development are summarized in Fig. 2. This figure graphically presents the difference in each of the enzymes measured between hatching (d 0) and first feeding (d 3.3 or 8 h into d 3) corresponding to the critical period for larval survival in this species. The differences between specific activity between d 0 and d 3.3 varied considerably among the enzymes assayed, and appeared to be hormone dependent. Larvae sampled from the TF-treatment group had a sharp increase in amylase activity by the time of first feeding, relative to the control group (Fig. 2). Aspartic protease activity was reduced when larvae were TF-treated, while activity increased slightly in the control group of larvae. Reduction of acid phosphatase activity was less in larvae with

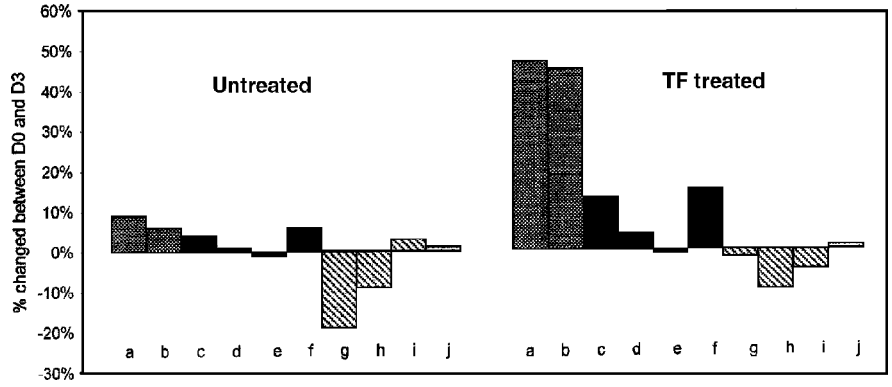



**Figure 2.** Inductive effect of the hormone treatment on changes in specific activity of digestive enzymes measured in larval threadfins, occurring between d 0 and d 3 after hatching. Percentage of change was the difference between the two values expressed as a percentage of the range of specific activities detected (see Methods and Materials). a: acid amylase, b: alkaline amylase, c: serine protease, d: collagenase, e: lipase, f: alkaline phosphatase, g: acid phosphatase, h: chitinase, i: aspartic protease, and j: cellulase.

the hormone treatment relative to larvae in the control group. Hormone treatment, however, did not affect specific activity of lipase.

## DISCUSSION

We have reported previously that a single hr of immersion in sea water containing a combination of $T_3$ and cortisol (TF) conveys survival benefits to Pacific threadfin during the larval period (Brown and Kim 1995). This effect has been attributed at least partially to the hormonal stimulation of the onset of gastrointestinal function (Kim and Brown 1997). We interpret these results as an indication that some hormone effects or interactions are sufficiently potent to override other variables responsible for differences in cohort survival (i.e., egg quality). An episodic increase in mortality was observed in three stages in the course of the experiment. Mortality occurred from the time of hatching (d 0) through the first feeding (d 5), immediately prior to notochord flexion (d 12 to d 14), and post-flexion prior to metamorphosis (d 20 to d 22).

These results are consistent with patterns experienced routinely in marine hatcheries involving a dietary shift. Survival was not quantified in this experiment, although we have seen a consistent and positive survival effect among the TF-treated groups over several years of experimentation (Kim and Brown 1997).

Specker (1988) has proposed a dietary supplement of thyroid hormones for preadaptation to prepare larval intestinal tissues for feeding, and Tanaka et al. (1995) demonstrated that $T_4$ treatment enhanced the thickness of epithelial cells of the alimentary tract, suggesting improved absorptive function. When larval *moi* were treated with a combination of $T_3$ and cortisol, the specific activities of most digestive enzymes increased prior to first feeding (Fig.1) suggesting that these regulatory hormones preconditioned the digestive tract for increased digestion (this study) and nutrient absorption (Kim and Brown 1997).

Amylases were relatively most elevated and certainly the most changeable digestive enzymes for larval *moi* during the first 2 wk of age. The TF treatment enhanced the specific activity levels of amylase at both pH 5.4 and 7.4 as well as serine protease activity (Fig. 1). These data suggest that a combination of $T_3$ and cortisol advanced the capacity of larval threadfin for prey digestion, which may have physiological benefits both in the first-feeding stage and in subsequent dietary shifts (e.g., the transition from the consumption of rotifers to *Artemia sp.*, during d 12 and d 13). The present results suggest that the net effects of hormone treatment consisted of both an advance in the timing of the ontogenetic pattern of amylases and serine proteases in the threadfin, and an increase in the specific activities of these enzymes particularly during the first few d after hatching.

Digestive enzyme activities increased at the time of mouth opening, even among starved larvae (Fig. 3), indicating that enzymes were produced prior to the presence of live feed in the gastrointestinal tract. During the first-feeding period (initiation of feeding until the point-of-no-return), amylase activity increased by more than threefold for the control group but decreased among starved larvae. This suggests some reliance during this stage on carbohydrate metabolism, possibly for energy, during the critical period. Specific activities of most digestive enzymes other than amylase were low even when prey (substrate) was abundant in the lumen. This indicates that protein and fat digestion are relatively unimportant during the first half of the larval period.

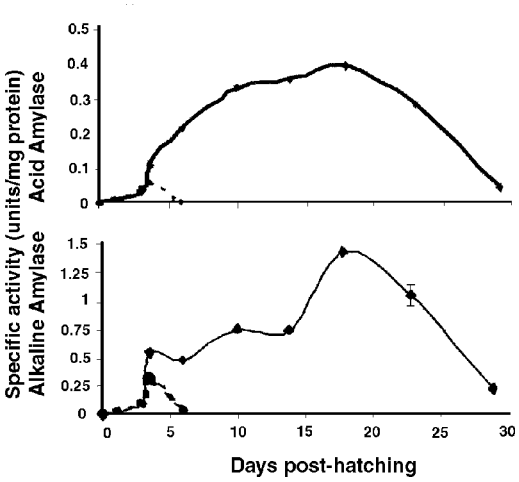



**Figure 3.** Effect of starvation on the ontogenetic patterns of digestive enzymes. Starved larvae are indicated by dashed lines (- - - -) and fed (controls) are shown as solid lines (__).

Nonetheless, when larvae were TF-treated at hatching, specific activities of lipase, protease, and alkaline phosphatase increased. In other words, digestive enzyme activities are inducible by hormones.

Improved protein and fat uptake by Japanese flounder treated with $T_4$ (Tanaka et al. 1995) and high absorptive function by cortisol-treated tilapia (Ayson et al. 1995) also support the maturation-promoting function on alimentary tissues of these hormones applied individually. Multiple hormonal regulators are known to interact in the control of gastrointestinal tract changes during amphibian metamorphosis (Dent 1988); cortisol and $T_3$ are among the most prominent, working in concert against the antimetamorphic actions of prolactin. Despite the lack of evidence to discriminate between possible direct and indirect actions of $T_3$ and cortisol on gut development and function, these hormones have been shown to have some direct peripheral interactions on developing target tissues (Redding et al. 1986; Vijayan et al. 1988; de Jesus et al. 1990).

Maternal deposit of cortisol into eggs and its importance in the regulation of embryonic development are still questioned (Brown and Bern 1989; Hwang et al. 1992; Tanaka et al. 1995; Tagawa 1996), but cortisol has been detected prior to larval corticosteroidogenesis (Perez et al. 1999). When both hormones are applied exogenously, as in this study, they appear to interact to promote one or more vital developmental processes, which can convey survival advantages even under compromised conditions.

The conclusion that the enzymes measured here reflect early digestive physiology appears consistent with other results, particularly when considered with evidence of development-promoting effects of these hormones at the same dosage, on the larval intestine in the same species (Kim and Brown 1997; Kim 1999). Nevertheless, because whole larvae were pooled for the extraction of enzymes, their specific tissue source can only be considered speculatively. It is possible, and in fact likely, that the early appearance of amylases and their promotion by thyroid hormone and cortisol is not restricted to the gastrointestinal system. Certainly these and other enzymes reported in this study could be involved in carbohydrate metabolism and tissue reorganization elsewhere in the developing larvae. More tissue-specific technical approaches such as histochemistry or *in-situ* hybridization may allow finer-resolution and more definitive examination of some of the ontogenetic questions raised in this study.

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